Tuberculosis (TB) is one of the world’s most prevalent diseases responsible for the largest fraction of infection related casualties.¹ The disease is caused by Mycobacterium tuberculosis a pathogen difficult to treat by standard antibiotics requiring therapies with a combination of drugs such as isoniazid, rifampicin, ethambutol and pyrazinamide. Moreover, in recent years a strong increase in multidrug-resistant (MDR) TB cases (resistant to at least isoniazid and rifampicin) was observed. It is estimated that around 9% of MDR TB samples were also extensively drug-resistant (XDR) TB, i.e., resistant to isoniazid and rifampin and to at least one representative of each class of the most effective second-line drugs (i.e., fluoroquinolones and the injectable drugs kanamycin, amikacin, or capreomycin).¹ This augments the challenge of treatment and requires new and more efficient drug development strategies. However, these are more challenged by a limited number of exploited targets and impaired uptake of small molecule drugs by the highly impermeable mycobacterial cell wall.²

M. tuberculosis is transmitted by aerosol and is initially taken up by alveolar macrophages, which phagocytose but do not kill the bacterium. Most infected individuals can immunologically control the disease. It is assumed that one-third of the world’s population is latently infected. The bacteria adapt and survive in diverse environmental niches in vivo, e.g. in solid granulomas, a characteristic feature of latent TB infection.³ It is presumed that M. tuberculosis resides in these regions in a slow growing or non-replicating, phenotypically drug resistant dormant-like state, due to limited availability and supply of oxygen and nutrients.³,⁴ During infection experiments it has been shown that M. tuberculosis accumulates triacylglycerols (TAGs) within intracellular inclusion bodies.⁵-⁷ The hydrolysis of TAGs to free fatty acids is an essential prerequisite for M. tuberculosis growth and survival requiring diverse lipases and hydrolases.⁶,⁸,¹⁰ During infection the liberation of fatty acids represents an important energy source¹¹ triggered by the availability of lipids in the host cell.¹² Moreover, it is believed that these fatty acids are crucial for bacteria to enter and maintain the dormant state by the production of foamy lung macrophages during latent infection.⁹,¹³ Accordingly, the M. tuberculosis genome encodes numerous hydrolytic enzymes involved in lipid metabolism.¹⁴ Among those, the lip gene family consist of 24 lipid/ester hydrolases termed Lip C to Z which share a conserved active site as well as alpha/beta hydrolase fold.⁶,¹⁵ Specifically targeting the bacterial lipid metabolism, could represent a viable strategy to address M. tuberculosis¹⁶ and open a new opportunity to shorten the currently required long-term TB therapy, when given simultaneously with known first and second line antibiotics.¹⁶ Thus focusing on known mammalian lipase inhibitors such as lalistat (La-0)¹⁷ and orlistat¹⁸ could represent a novel therapeutic strategy inhibiting TAG metabolism. Despite their prominent role only little is known about the exact function of lipid hydrolysing enzymes in mycobacteria. Orlistat was previously shown to reduce viability and mycolic acid biosynthesis for numerous mycobacterial strains.¹⁹ In depth target analysis via activity based protein

Human lysosomai acid lipase inhibitor lalistat impairs Mycobacterium tuberculosis growth by targeting bacterial hydrolases†‡

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Lalistat inhibits growth of Mycobacterium tuberculosis in bacterial culture as well as in infected macrophages. Target identification by quantitative proteomics revealed a cluster of 20 hydrolytic proteins including members of the lipase family. Lipases are essential for M. tuberculosis fatty acid production and energy storage thus representing promising antibiotic targets.
potency of previously studied orlistat e.g. for the treatment of obesity.23,24 The beta-lactone orlistat karyotic and eukaryotic organisms. Several compounds have eral bacterial lipases were identified suggesting inhibition of infection is treated by a combination of up to four dif-
markably, a strong effect was obtained for the combined ap-
pound exhibited a MIC of 25
exhibit MIC values in the range of 0.04 to 1.0
was utilized as a common precursor for lalistat (La-0) and its
to the Lip family. Orlistat or related hydrolase inhibitors that
electronic properties of La-0 we anticipated binding to di-
from incoming lipoproteins.24 Lalistat, similarly to orlistat,
binds to the active site serine of lipases covalently and
thereby inhibits enzyme activity.27,28 Given the structural and
electronic properties of La-0 we anticipated binding to di-
verse lipases which may deviate from the orlistat target spec-
ment and complement mechanistic insights. We thus
spected if lalistat impairs viability of M. tuberculosis by se-
several independent approaches.

First, minimal inhibitory concentrations (MICs) were de-
termined by addition of La-0 to diluted cultures of M. tubercu-
losis H37Rv and utilizing the resazurin assay.29–31 The com-
 pound exhibited a MIC of 25–50 μM (Fig. S1‡) exceeding the poten-
cy of previously studied orlistat ∼30 μM.19 Currently ap-
plicated standard therapeutics such as rifampicin and isoniazid
hibit MIC values in the range of 0.04 to 1.0 μM.32 M. tubercu-
losis infection is treated by a combination of up to four dif-
ferent drugs; we therefore tested mixtures of La-0 and various
(myco-)bacterial drugs on two-dimensional MIC plates. Re-
markably, a strong effect was obtained for the combined ap-
plication of lalistat and vancomycin resulting in a MIC drop
of factor 4 for lalistat and 16 for vancomycin (Fig. 1A). Using
the fractional inhibitory concentration index (FICI)33,34 a co-
operative effect could be concluded as both molecules inter-
fer with the composition and assembly of the cell wall. Fur-
thermore, growth of green fluorescent protein (GFP) ex-
pressing M. tuberculosis H37Rv was monitored for 7 days in
presence of either La-0 or rifampicin (control) as described
by Michelucci et al.35 Interestingly, growth inhibition was
observed for lalistat down to a concentration of 4 μM
(Fig. 1B). Finally, the effect of La-0 on intracellular growth of M. tuberculosis in human macrophage host cells was deter-
mined. To this end we infected human monocyte derived
macrophages with strain M. tuberculosis H37Rv (multiplicity
of infection 1:1) for 4 h and subsequently cultured the cells
for 7 days with various concentrations of La-0 and DMSO
(control). Cells were then lysed and CFUs were determined as
described previously.36 Again lalistat substantially reduced
bacterial load by 55% compared to the untreated control
suggesting that the compound can even address intracellular
bacteria (Fig. 1C). These results point towards an essential
mechanism addressed by lalistat and given its specificity for
lipases a link to essential metabolic processes was subse-
quently evaluated by in situ target identification.

To analyse the mode of action we identified lalistat pro-
tein targets in live M. tuberculosis cells via ABPP. For this
technology a chemical probe bearing an alkyne tag (La-1) was
synthesized following established protocols (Fig. 2A).27 In
brief, a piperidine moiety was introduced in commercially
available 3,4-dichloro-1,2,5-thiadiazole, replacing one of the
chlorine substituents while the other was subsequently
converted into an hydroxyl-function using potassium hydrox-
ide (Scheme 1). The newly synthesized hydroxy-heterocycle
was utilized as a common precursor for lalistat (La-0) and its
alkynylated probe (La-1) using piperidyl chloroformate or
4-ethylpiperidine chloroformate, respectively, to form the es-
sential carbamate functionality.

Satisfyingly, La-1 retained activity and reduced M. tubercu-
losis growth comparable to the parent molecule La-0 (Fig. S1‡).

Fig. 1 Lalistat inhibits M. tuberculosis growth. (A) Two-dimensional MIC determination. Lanes: La-0 dose-down. Rows: vancomycin dose-down. (B) Inhibition of GFP-expressing M. tuberculosis H37Rv with rifampicin (left) and lalistat (right). All experiments were performed together with the same DMSO control (identical in both graphs). (C) Colony forming units (cfu) of M. tuberculosis H37Rv recovered from human macrophages in presence and absence of inhibitors. Shown is one representative experiment out of two performed, each consisting of three (B) and two (C) technical replicates.
Next, target identification experiments were conducted. *M. tuberculosis* cells were grown to log phase and incubated with La-1 or DMSO as a control. After cell lysis a rhodamine tag was attached to the alkyne moiety of La-1 via bioorthogonal click-reaction\(^\text{37-39}\) to visualize target proteins on SDS-PAGE gel by fluorescence scanning. Protein labelling at different probe concentrations are displayed in Fig. 2B. Interestingly, proteins around a molecular size of 30 kDa showed increased fluorescent signal response with raising probe concentration while others (∼50 kDa) only marginally changed intensity. A concentration between 30 μM and 60 μM was sufficient to achieve saturated labelling. We thus proceeded with gel-free proteomics and performed labeling studies with 30 μM La-1 in *M. tuberculosis* H37Rv to unravel the identities of target proteins. For these studies a biotin tag served as a handle to enrich labeled proteins on avidin beads (Fig. S2‡). After on-bead digestion primary amines were modified by heavy, medium and light isotopes using dimethyl labeling (DL).\(^\text{40}\) Labelling ratios of probe versus DMSO were normalized, z-score and log\(2(x)\) transformed. Target proteins with a high...
enrichment and statistical significance (see ESI‡ for details) are located in the upper right area of the enrichment volcano plot in Fig. 2C. To validate these enriched hits we performed competition experiments where cells were pre-incubated with La-0 (60 μM) and subsequently treated with 30 μM La-1 probe (Fig. 2C). Strikingly, spectra of enrichment and competition experiments include 7 Lip enzymes (LipN, -I, -R, -M, -G, -T and -O) that selectively react with orlistat.

A comparison of lalistat hits with results of previous orlistat proteome labeling studies in M. bovis (BCG) revealed Lip M, O, N, I and G as shared targets. Interestingly, while Lip R and T were selectively captured by orlistat-like probes suggesting a complementary target profile by both lipase inhibitors due to their different physico-chemical properties. It is thus intriguing to speculate that the shared preference for a common set of Lip enzymes may contribute to the growth inhibitory effects of both compounds.

LipR as one of the most strongly enriched hits by La-1, was exemplary confirmed as target by a heterologous expression and subsequent labeling with the probe. A strong fluorescent band indicated specific binding (Fig. 3). Beside members of the Lip family we identified six additional enzymes with lipolytic activity: Rv0183, a highly conserved monoacylglycerol lipase, Rv1984 (ref. 43 and 44) a cutinase with lipolytic activity: Rv0183, a highly conserved monoacylglycerol lipase, Rv0045c (characterized esterase) and Rv2715, Rv1192, Rv1399c (uncharacterized proteins Rv2715, Rv1192, Rv1399c (characterized esterase) and Rv0045c which are suggested to participate in lipid hydrolysis in mycobacterial metabolism.

We also detected few putative non-lipolytic enzymes bound by lalistat: Rv1367 and Rv1730, both possibly involved in cell wall biosynthesis, as well as amidas (Rv2888c and Rv2363), iminopeptidase (Rv0840c) and two proteins of unknown function (Rv0293c, Rv1191). To date it is not clear whether mycobacterial lipases hydrolysing short-chain substrates, like LipR, would participate in non-lipolytic metabolic processes because it remains unknown whether they have additional phospholipase, thioesterase or protease activity.49

Conclusions

Taken together, the specificity of lalistat for a suite of mycobacterial lipases is intriguing. Given the importance of these enzymes for M. tuberculosis viability during infection they may represent promising drug targets. Future studies need to further dissect and characterize the exact function and mechanism of these enzymes in order to design customized inhibitors suited to interfere with essential metabolic processes specifically in the bacteria. Here, orlistat and lalistat probes represent interesting chemical tools that allow simultaneous and selective detection of lipase activities in living mycobacteria. With regard to the paucity of potent antimycobacterial inhibitors future tuberculosis treatment approaches could largely benefit from adding these compounds into treatment regimens. They could also pave the way for the development of more specific drugs targeting mycobacterial lipases. Their use in combination with known antimycobacterial agents may offer an urgently needed opportunity to improve and shorten TB therapy.

Acknowledgements

The work was funded by the Deutsche Forschungsgemeinschaft (DFG) SI1096/6-1 to SAS. JL was supported by the Studienstiftung des deutschen Volkes. NR was funded by a grant of the DFG Excellence Cluster EXC306 “Inflammation at Interfaces”. KK was supported by a doctoral fellowship of the Verband der Deutschen Chemischen Industrie e.V. (VCH). We gratefully acknowledge Lisa Niwinski and Carolin Golin (FZ Borstel), Mona Wolff and Katja Bäuml (TUM) for excellent technical support. We thank Eric Rubin for helpful advice and support.

Notes and references

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